

# Principles and Applications of Biological Membrane Organization

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## Keywords

Biological membrane, lipid biophysics, phase separation, membrane heterogeneity, membrane organization

## Abstract

Many critical biological events, including biochemical signaling, membrane traffic, and cell motility, originate at membrane surfaces. Each such event requires that a specific group of proteins and lipids rapidly assemble together at a specific site on the membrane surface. Understanding the biophysical mechanisms that stabilize these assemblies is critical to decoding and controlling cellular functions. Here we review progress toward a quantitative biophysical understanding of the mechanisms that drive membrane heterogeneity and organization. We begin from a physical perspective, reviewing the fundamental principles and key experimental evidence behind each proposed mechanism. Then we shift to a biological perspective, presenting key examples of the role of heterogeneity in biology and asking which physical mechanisms may be responsible. We close with an applied perspective, noting that membrane heterogeneity provides a novel therapeutic target that is being exploited by a growing number of studies at the interface of biology, physics, and engineering.

## 1. Introduction

The membrane interfaces of eukaryotic cells are highly complex, containing tens of thousands of distinct lipid species (132), and thousands of distinct membrane proteins (3). Despite this complexity, biological processes that occur at membranes, such as receptor signaling, cell-cell communication, and membrane traffic, typically rely on just a handful of critical protein and lipid constituents. These critical constituents assemble within seconds to minutes. The ability of membrane components to assemble rapidly to form functional complexes suggests that membranes may be spatially organized, reducing the entropic and kinetic barriers to molecular assembly. The reliance of biological functions on membrane organization is widely recognized and increasingly studied (3, 84, 123). Recent discoveries have made progress toward understanding both the physical mechanisms that organize membranes and the biological functions of membrane organization.

Here we review these recent discoveries and the general biophysical principles that are emerging from them. The first section evaluates current understanding of the fundamental mechanisms that organize membranes. We begin with chemical mechanisms, which arise from affinity between specific groups of proteins and lipids. Then we consider physical mechanisms, which arise from the combination of chemical affinity and non-specific molecular interactions. Such mechanisms include lipid and protein phase behavior, the impact of membrane curvature on protein and lipid partitioning, and mechanisms that rely on the suppression of membrane fluctuations. The second section of this review discusses how these fundamental mechanisms contribute to biologically-relevant examples of membrane organization, which include assembly of membrane buds and signaling complexes, inter-organelle contact sites, and cell-cell contacts. Finally, in the last section we consider membrane organization as a novel therapeutic target and an inspiration for the design of biomimetic drug delivery systems.

## 2. Mechanisms of Membrane Organization

We begin by introducing the fundamental mechanisms that have been demonstrated to organize biological membranes, Figure 1. These mechanisms are intriguingly diverse, spanning length scales from single molecules to micrometers, and drawing on interactions between lipids, proteins, and lipid-protein complexes.

### 2.1. Chemical Mechanisms

Spatial heterogeneity can arise if some part of the membrane is presented with a target to which one or more membrane components can specifically bind. The chemical potential of the relevant membrane component or components will depend on whether it is bound to the target and, therefore, located next to the target. Membrane constituents that bind to a target will each have their chemical potential  $\mu$  reduced by the bond energy.

A system will spontaneously change to minimize its free energy, as the state of minimum free energy is the equilibrium state. The change in free energy,  $\Delta E$ , depends on both

enthalpic and entropic terms. For biological systems and processes, any change in the volume of a constituent  $\Delta V$ , is negligible and therefore so is  $P\Delta V$ , where  $P$  is the bulk pressure in the system. Therefore, we omit the  $P\Delta V$  term from a description of the change in free energy. The change in enthalpy describes a change in internal energy  $\Delta U$ . The internal energy of the membrane depends on the chemical potentials of its constituents, Equation 1.

$$U \propto (\sum_i \mu_i n_i) \quad 1.$$

The change in Helmholtz free energy is given by Equation 2.

$$\Delta E = \Delta U - T\Delta S \quad 2.$$

In Equation 1,  $\mu$  is the chemical potential of a constituent,  $n$  is the number of that constituent present in the system, and  $i$  is an index designating the specific constituents. The quantity  $\Delta(\sum_i \mu_i n_i)$  gives the weighted sum of the change in chemical potentials upon binding. In the notation we use here, the same chemical constituent must be indexed differently depending on whether it is bound or unbound. Clustering together one or a few membrane constituents at a target site will reduce the mixing entropy  $S$  of the system, which Equation 2 shows will incur a temperature-dependent change in free energy of the form  $-T\Delta S$ . The size of  $\Delta S$  depends on the number of molecules that have been spatially segregated. If the changes in chemical potential, and therefore the binding energies, are sufficiently great such that  $|\Delta(\sum_i \mu_i n_i)| > |T\Delta S|$ , the system's free energy will be minimized by binding that creates spatial segregation. This behavior has been widely demonstrated in experiments on model membranes (2, 4, 43, 44, 79, 119, 121).

These simple molecular principles play an important role in each of the more complex mechanisms described in the next section. Further, chemical recognition and the assembly of biochemical scaffolds are critical to many examples of membrane heterogeneity in the cell, as described in the next section of this review.

## 2.2. Physical Mechanisms

This section summarizes mechanisms by which non-specific physical interactions, often in combination with chemical binding as discussed above, can give rise to membrane ordering.

### 2.2.1. Lipid Phase Separation

The phenomenon of lipid phase separation arises because different lipids, and even a single lipid species, can adopt multiple phases (25, 34). There is a great deal of literature on lipid phase behavior. At the melting temperature,  $T_m$ , a membrane consisting of a single lipid species will transition from an ordered solid or gel phase to a disordered fluid phase, in which lipids have conformationally-disordered tails and diffuse rapidly in the plane of the membrane. If a membrane is composed of two or more species of lipid or sterol and the temperature is greater than the highest  $T_m$  of any species in the mixture, then the membrane will be homogeneous. However, if the membrane is cooled to a

temperature that is lower than the highest  $T_m$  of any lipid in the mixture, then the membrane may become heterogeneous through phase separation (81).

The change in free energy with phase separation,  $\Delta E$ , can be described using Equation 2. Here the internal energy,  $U$ , represents interactions between adjoining lipids, rather than chemical potential. The weight of the entropic term,  $T\Delta S$ , scales with temperature  $T$ . At sufficiently high temperatures,  $E$  is minimized when the system is well-mixed. As the temperature is decreased, the system will enter a range of temperatures at which  $E$  is minimized by clustering together lipids in two or more distinct phases. The highest such temperature is the demixing transition temperature,  $T_{\text{demix}}$ .

At equilibrium, the maximum number of different phases that may co-exist,  $P$ , is given by the Gibbs phase rule, Equation 3.

$$P = C - F + 2 \quad 3.$$

Here,  $C$  is the number of chemically-distinct constituents and  $F$  is the number of degrees of freedom of the system.  $F$  is the number of intensive variables that can vary independently. Composition, temperature, and pressure are all intensive variables. In all experiments of which we are aware, the system is closed to changes in membrane composition. Fixed membrane composition negates one potential degree of freedom. There have been few reports of experimental work where lateral membrane pressure is controlled (11, 57, 111), so here we will consider only cases in which pressure varies freely. If temperature and pressure can vary independently, then  $F = 2$  and  $C = P$ . A membrane composed of a large number of lipid and sterol types has a high value for  $P$  and, in principle, the potential for many different phases to coexist at equilibrium across a range of temperatures and pressures. In contrast, a membrane composed of only one type of lipid has  $P = 1$  and only one phase can exist at equilibrium across a range of temperatures and pressures. For such a single-component membrane, phase coexistence is possible only when the temperature is fixed at the melting temperature.

A mixture of a low-melting lipid, a high-melting lipid (or mixture of high-melting lipids), and a sterol is commonly used as a model system for the cell membrane, Figure 2A. Such mixtures have been widely observed to phase separate into regions of two coexisting liquid phases - a liquid-ordered phase and a liquid-disordered phase (7, 11, 14, 15, 17, 23, 36, 37, 47, 59, 66, 72, 74-77, 80, 88, 92, 97, 98, 128, 129, 134, 136, 138, 140, 142, 147-150, 154). These two phases have different compositions, given by empirically-determined tie-lines on a ternary phase diagram and the points at which these tie-lines touch the phase boundaries. The Gibbs phase rule also allows for the coexistence of three phases in a three-component mixture - for example, a liquid-ordered phase, a liquid-disordered phase, and a solid phase (99, 139).

For ease of representation on two-dimensional surfaces, it is common to show ternary phase diagrams as triangles, with each axis representing the proportion of one chemical constituent, Figure 2A. As temperature increases, the entropic contribution to free energy becomes increasingly more significant and the demixing region decreases in size. Within

the demixing region, at a fixed temperature, horizontal tie-lines will get shorter as the composition approaches the critical point (141). By extrapolating to find the composition at which the tie-line would have zero length, the critical point at that temperature can be identified. As this use of tie-lines implies, at a critical point the composition of the liquid-ordered and liquid-disordered phases is identical. Near a critical point the composition of the two phases is very similar. At a temperature just below a critical point, the interfacial energy, or line tension, between the two phases is small and therefore domains of the two phases have large fluctuations due to the thermal energy at room temperature, rather than being rounded as a result of the higher line tensions found farther from a critical point (67). At a temperature just above a critical point, there are transient fluctuations in composition and phase, although the time-averaged membrane is homogeneous. Experiments have shown that the shape and composition fluctuations seen in ternary lipid membranes near a critical point is well-described by the 2-D Ising model (66).

Systems at or near critical points are very sensitive to small perturbations in intensive variables. For a living system, this could correspond to a rapid or fine-tuned response to an input. In this light, it is interesting that experiments have shown that membrane extracts from living cells are near a critical point at physiological temperature (137).

#### 2.2.2. Protein phases: solid lattices and emerging 2D liquids

Many processes, from vesicular budding to metabolic and signaling pathways, occur at discrete sites on cell membranes. Dozens of transmembrane and peripheral membrane proteins may be required to carry out a single common pathway. To spatiotemporally compartmentalize these processes on the membrane surface, proteins assemble into dynamic, often transient clusters. Despite extensive biochemical characterization of membrane protein assemblies, the physical basis for their formation has often been overlooked. Over the past decade, liquid-liquid phase separation (LLPS) has emerged as a mechanism to selectively localize components of a common pathway from a free monomeric state to a liquid condensed state. In the three-dimensional space of the cellular cytosol, LLPS drives the formation of micron-scale “membraneless organelles” (18, 20). However, there is increasing recognition that LLPS can also drive nano- to micron-scale protein assembly on membranes. Indeed, restricting proteins to two dimensions lowers the critical concentration for phase separation to occur. This property could serve as a convenient way to confine assembly of cytosolic factors to the membrane without permitting their assembly in solution (29).

Proteins can separate into two phases – a protein-rich liquid phase (G), and a protein-poor dilute phase (L), Figure 2B. Two-dimensional LLPS has been identified in several systems, including T cell receptor signaling (70, 130) and cell adhesion receptor signaling (9). Multivalency is a key feature of these networked protein clusters, and is often generated through modular repeats, such as PRD and SH3 domains. Several multivalent domains, even if weakly interacting, can promote protein-protein over protein-solvent interactions to drive phase separation (8). Another feature commonly associated with these networks is the presence of intrinsically disordered protein (IDP) domains (8, 31). IDP domains allow recognition motifs within them, such as phosphorylated tyrosine, to

remain exposed to modular binding domains. These domains also provide conformational flexibility, which helps the system to remain fluid (60).

Phase separated protein assemblies on membranes have been described for only a few sets of proteins thus far. However, the multivalency of the membrane proteins that have been shown to phase separate is a common feature of the multiprotein systems involved in many other membrane signaling, trafficking, and metabolic pathways (29). Therefore, it is likely that LLPS contribute to the compartmentalization of many other membrane-localized assemblies.

### 2.2.3. Compartmentalization by Membrane Curvature

A growing body of literature is documenting the ability of proteins and lipids to sense curved membranes (5, 12, 55). This phenomenon arises from preferential molecular partitioning to membrane regions of specific curvature. As a result of this effect, curved regions take on unique molecular compositions, effectively organizing the membrane. Both lipids and proteins have been shown to participate in curvature-sorting phenomena.

#### 2.2.3.1. Lipid Curvature Sorting

The ability of lipids to partition selectively to regions of distinct membrane curvature has been the subject of several excellent reviews (12, 55). The earliest observations of the ability of membrane lipids to segregate into regions of distinct curvature was based upon the bulging of lipid domains from the surfaces of giant unilamellar vesicles (13), Figure 3A. Specifically, in membrane vesicles that separated into liquid-ordered and liquid-disordered phases, the minority phase was found to pucker outward, taking on a higher curvature relative to the majority phase and thereby creating a bumpy appearance. This phenomenon arises from minimizing the mechanical energy of the membrane, which is given by a competition between the total line energy at phase boundaries and the total bending energy of the membrane (6, 13). Line energy, the one-dimensional analogue to two-dimensional surface tension, arises from hydrophobic mismatch along the phase boundary (49). Bulging membrane domains naturally repel one another, since their approach would require the regions of the membrane between them to take on a high curvature in the opposite direction (105). This repulsion can order the membrane surface, setting up a periodic patterning of the two phases over length scales of tens to hundreds of microns, much larger than the dimensions of individual lipids (114).

In addition to these line tension-driven effects, the bending mechanics of the lipid phases can also give rise to curvature sorting. This effect has been nicely demonstrated by mechanical pulling of lipid tethers from giant unilamellar vesicles (64) and pulling by actomyosin networks assembled in vitro (112). In these experiments, the softer liquid-disordered phase partitions preferentially to the highly-curved tether, minimizing the total mechanical energy of the system (105). Similarly, for supported lipid membranes formed on ridged supports, the stiffer liquid-ordered phase partitions preferentially to areas of lower curvature (106). These effects involve large ensembles of lipids and typically range from sub-micrometer to hundreds of nanometers in dimension. In contrast to these long

length-scale mechanisms, individual lipids display curvature preferences that are too weak to drive substantial differences in partitioning between membranes of different curvature (133). However, as discussed in the next paragraph, many membrane binding proteins display curvature preferences at the molecular scale.

#### 2.2.3.2. Protein Curvature Sorting

Similar to lipids, proteins display preferences for regions of the membrane with distinct curvatures, Figure 3B. Here much of the attention has focused on two primary mechanisms: insertion of amphipathic helices into curved membranes, and shape complementarity between proteins and curved membranes (5). In the first mechanism, amphipathic helices, sometimes referred to as ALPS (amphipathic lipid-packing sensor) motifs (16), are thought to insert into membrane defects. Such defects appear to be more abundant on highly curved membrane surfaces (62), as revealed by an assay in which amphipathic helices were allowed to partition among populations of substrate-tethered vesicles with diameters ranging from 20 to 200 nanometers.

Interestingly, the affinity between helices and defects was not found to be a strong function of curvature in these studies, likely because the nanometer-scale defects are small in comparison to the membrane radius of curvature, which is still tens of nanometers for the most highly curved structures. In addition to amphipathic helices, proteins with inherently curved membrane-binding surfaces, such as the members of the BAR (Bin/Amphiphysin/Rvs) domain superfamily (107, 126) and dynamin (93), are thought to bind preferentially to membrane surfaces that match their curvature. Similarly, transmembrane proteins with asymmetric, wedge-like shapes, such as the KvAP potassium channel (1) and the dopamine transporter (27) have been demonstrated to partition preferentially to membrane tubules and filopodial protrusions, respectively.

#### 2.2.4. Fluctuation Suppression - Membrane Organization by Adhesion Sites

Like other soft materials, lipid bilayer membranes are subject to fluctuations in spatial position, arising from thermal energy, according to the Fluctuation-Dissipation Theorem (90, 110). Adhering the membrane to another material will increase the energetic cost for a fluctuation of a given magnitude, and therefore will reduce the overall distribution of undulation sizes. The degree to which adhesion reduces fluctuations depends on both the material to which the membrane adheres and the way adhesion links the membrane to the other material. For example, adhesion to a rigid material suppresses fluctuations more than adhesion to another lipid membrane. Similarly, direct bonding or absorption to the substrate material suppresses fluctuations more than linking to the substrate through a floppy polymer.

We have previously proposed a mechanism by which suppressing undulations might lead to demixing in a lipid membrane (53), Figure 4. The undulations in the membrane contribute to its entropy by increasing the number of microstates  $W$ , since  $S$  is proportional to  $\ln W$ . Reducing the magnitude of these undulations reduces  $W$  and therefore reduces the size of the  $S$  term in the Helmholtz free energy, as described by

Equation 2. This effect will increase the relative importance of the internal energy term,  $U$ , and therefore favor demixing. Therefore, we expect that suppressing membrane undulations by other mechanisms, such as membrane tension, ought also to impact demixing. Published studies examining the impact of membrane tension on lipid phase separation have not achieved consensus (57, 111). We have previously suggested that this discrepancy might be explained by difference between the two experiments, which used two different methods to increase membrane tension. As such they may have accessed two different regimes of membrane tension; 1. undulation suppression at lower tension and 2. increased inter-lipid spacing at higher tension (42, 54). This interpretation remains speculative at this time.

#### 2.2.5. Cytoskeletal Compartmentalization

The cytoskeleton's involvement in membrane compartmentalization can be described using the membrane-skeleton fence model, where the actin-based, filamentous cytoskeleton forms a mesh-like network on the cytoplasmic surface of the plasma membrane (83, 85). This network confines transmembrane proteins by sterically interacting with their cytoplasmic domains (40, 41), thus corralling them into distinct compartments with widths that can range broadly from 10 to 200 nm (83, 85). These compartments have been identified using single particle tracking methods (86, 116) and have recently been visualized using super-resolution microscopy (145). This model became apparent as multiple researchers observed that diffusion coefficients for transmembrane proteins on the plasma membrane were 5-50 times smaller than coefficients for the same protein on artificial, reconstituted membranes (83). These reductions in diffusion coefficients are attributed to an anomalous type of diffusion known as "hop diffusion" (83, 85), where proteins exhibit Brownian diffusion within a single compartment, but slowly jump between adjacent compartments over time. These proteins can only undergo jumps when thermal fluctuations in the membrane create an opening between the membrane and the cytoskeleton or when the membrane and cytoskeleton temporarily dissociate as a result of dissociation-association equilibria.

Lipids on the extracellular leaflet of the plasma membrane have also been observed to undergo hop diffusion (48), which was a surprising result at the time since these lipids do not interact with the skeleton directly. To rationalize this observation, an additional model was proposed – the anchored-transmembrane protein picket model (83). In this model, transmembrane proteins become anchored to the skeleton fence and line up along it, effectively serving as rows of pickets. These pickets inhibit free diffusion of lipids due to steric hindrance and hydrodynamic friction-like effects with the immobilized transmembrane proteins (24).

Due to hop diffusion, monomeric components are able move between compartments freely. However, when molecules form assemblies, such as signaling-induced clusters, they become trapped within the compartments (83). If cytoskeleton fences were not present, clustered molecules would diffuse around the membrane freely, making it difficult to localize signaling responses to the areas in which they were received. Therefore, the



fences and pickets models provide a rationale for the ability of the cytoskeleton to spatiotemporally organize molecules on the membrane surface.

### **3. Biological Examples of Membrane Organization**

This section introduces key biological structures that require proteins and lipids to organize on membrane surfaces. For each example we review evidence for the underlying mechanism or mechanisms thought to be responsible for stabilizing membrane heterogeneity.

#### **3.1. Membrane buds**

Curved membrane structures are ubiquitous within the cell and vital to cellular function and physiology (33, 100). However, lipid bilayers themselves are inherently resistant to deformation. To achieve membrane remodeling, cells employ proteins that are capable of sensing and generating membrane curvature. In most cases, these proteins consist of heterogeneous assemblies.

One example is evident in clathrin-mediated endocytosis (CME) (33). During CME, cytosolic proteins such as AP2 detect binding motifs that are present on the intracellular side of the transmembrane receptors (21). Once the motif is bound, a myriad of additional cytosolic, multivalent proteins bind to the anchored protein, as well as to one another. A fraction of these proteins also bind to the membrane surface via protein-lipid interactions. This assembly results in a concentrated network of proteins on the membrane surface that recruits clathrin and promotes its assembly. This network also stabilizes convex curvature of the underlying plasma membrane (100).

The ability of endocytic proteins to sense membrane curvature assists in their ability to assemble on curved surfaces. Curvature sensing structures, such as amphipathic helices and BAR domains, are present among many of the CME adaptor proteins, including AP180 (102), Epsin1 (46), and Amphiphysin1 (107). These proteins also contain large IDP domains, which have recently been shown to contribute to curvature sensing (151, 152). Moreover, CME is just one example of a cellular process where organized protein networks interact with the plasma membrane to generate curvature. Other examples include viral budding (71), in which viruses utilize the host cell's ESCRT machinery to generate plasma membrane protrusions, and cytokinesis (101), in which specialized protein machinery promotes fission of the plasma membrane between nascent daughter cells.

#### **3.2. Signaling assemblies**

Lipid phase separation is a physical mechanism that is important for cell signaling. Specifically, lipid rafts have been proposed to play an integral role in many signal transduction pathways (89, 125). These lipid domains are enriched in sphingolipids and cholesterol (124), adopting a liquid-ordered phase. The functionalities of various receptors are thought to rely upon their partitioning into lipid rafts, where they form

supramolecular assemblies upon activation. Examples include G protein-coupled receptors (GPCRs) (19, 32), immune receptors (50, 122), receptor tyrosine kinases (109, 118), apoptotic receptors (26, 65) and chemotaxis receptors (51, 91). Upon assembly, these signaling clusters can be corralled within cytoskeletal compartments through a phenomenon known as oligomerization-induced trapping. Here membrane fences entrap receptor assemblies more effectively than they entrap monomeric receptors (82). By spatially confining the assemblies as they are formed, this entrapment allows the cell to sense the location from which the extracellular signal was received. This type of localization is essential for polarized signaling processes, such as cytoskeleton reorganization or chemotactic cell migration (82).

Another biological example of organized signaling assemblies are nano- and micron-scale liquid condensates that are formed through LLPS. Recently, it was discovered that the transmembrane cell adhesion receptor nephrin binds cytosolic proteins Nck and N-WASP to assemble into liquid clusters on the membrane (9). This network then promotes local reorganization of the actin cytoskeleton.

### 3.3. Immunological synapse

LLPS on the membrane has been well studied in the context of T cell activation. Upon activation, T cell receptors trigger the phosphorylation of the transmembrane protein Lat. The multiple phosphorylated tyrosine residues on Lat then bind multiple copies of the adaptor Grb2. In turn, each Grb2 provides two binding sites for the Ras GEF Sos1 (68). The multivalency of this interconnected lattice results in liquid-like clusters that effectively enhance the ability to recruit downstream effectors (70). This protein phase separated network may also be coupled to lipid domain formation, as Sos1 requires anionic lipids for its activation (56, 69).

In vitro, Grb2 and Sos1 trigger the coalescence of phosphorylated Lat into clusters, while Lat dephosphorylation dissolves these clusters (70, 130). The phosphatase CD45 is excluded from Lat:Grb2:Sos1 clusters, while other effector proteins are enriched there. This system illustrates an important function of liquid-liquid phase separation: to dictate through interaction preference which proteins to incorporate or exclude in the condensed phase(39). A steric mechanism also works to drive CD45 from activated T cell membrane clusters. As membrane receptors tightly couple the T cell and antigen-presenting cell, the closely apposed membranes sterically exclude the large extracellular domain of CD45, resulting in its spatial partitioning away from the interface (28).

### 3.4. Inter-organelle contact sites

Membrane contact sites (MCSs) are sub-micron domains formed between closely tethered organelles. These contacts can be stable, but are often dynamic, transient, and mobile (108, 113). Most MCSs organize several types of proteins at a single locus. Typically, these proteins function to tether the membranes, transfer material between organelles, or scaffold a multiprotein complex. Other proteins determine the composition

of the MCS by driving recruitment or exclusion of proteins with certain curvature, electrostatic, or hydrophobic preferences (120).

Membrane lipid composition is tightly intertwined with MCS structure and function. Lipid biosynthesis and transfer between membranes occur at many different MCSs. Accordingly, MCSs are often enriched in certain lipids, and in some cases may be defined by the presence of lipid domains (95, 103). At contacts between the ER and mitochondria, membranes contain lipid raft-like sterol and ceramide rich domains (63, 117). These contacts also tend to be in close proximity to assemblies of lipid biosynthesis metabolons, suggesting that it may be efficient to spatially couple lipid synthesis to the MCSs that use and regulate those lipids (131).

The physical mechanisms that drive MCS formation and organization largely remain unknown. For example, what factors dictate the distance between membranes or the lateral extent of their contacts? How are functionally and compositionally distinct MCSs between the same organelles arranged and maintained? The answers to these questions will provide valuable insights into how MCS organization influences function.

### 3.5. Cell-Cell contacts

Intercellular junctions play key roles in coordinating mechanical and chemical communication between neighboring cells. In accordance with these functions, the membrane surfaces involved in forming junctions are enriched in specific proteins and lipids. For example, gap junctions consist of hexameric pore assemblies of connexin proteins (52). When two such pores on the surfaces of neighboring cells dock with one another, a channel is opened that allows passage of small molecules from the cytoplasm of one cell to diffuse directly into the cytoplasm of the neighboring cell (94). At the interface between cells, gap junction pores assemble into dense, regular arrays that can span many microns (58). Assembly of these gap junction plaques is thought to arise from physical effects rather than biochemical binding events. Specifically, since the membranes of neighboring cells have to bend toward one another to form gap junction, tight packing of multiple junctions is thought to be energetically favorable because it minimizes the overall curvature energy of the membrane (22).

In contrast to gap junctions, tight junctions, which rely on occludins, claudins, and other adhesions proteins, support the barrier functions of cellular layers by preventing molecular diffusion through the extracellular space between two neighboring cells (135). Here biochemical assembly of junction proteins into linear structures creates barriers that can span the entire cell-cell contact zone (127). Lastly, adherens junctions, which provide cellular layers with mechanical integrity and support mechanical signaling, have more complex molecular compositions than the other two types of junctions (61). Here cadherin and catenin membrane proteins organize the assembly of the cellular cytoskeleton on length scales that span multiple cells. Interestingly, gap junctions (38), tight junctions (104) and adherens junctions (30) have each been found to be enriched in cholesterol and other lipid raft-associated lipids, suggesting that these structures may be reinforced by co-assembly of liquid ordered membrane phases.

## 4. Opportunities for Therapeutic Intervention

Here we summarize several emerging areas in which the heterogeneity of the membrane provides either a model or a target for an engineered therapeutic system. This field provides a fertile area for future application of the growing biophysical understanding of membrane heterogeneity.

### 4.1. Phase Separation of Drug Carriers

Fusion of synthetic lipid vesicles with cellular membrane surfaces is a promising approach for delivering therapeutics to target cells. Membranes containing positively charged lipids, such as DOTAP (1,2 dioleoyl-3-trimethylammonium-propane), can effectively fuse with negatively charged membranes (96, 143). In practice, large concentrations of fusogenic molecules are often necessary for macromolecular delivery, which leads to toxicity and immunogenicity in vivo (45). To circumvent this obstacle, we have recently proposed the use of phase separated liposomes (73).

Using liposomes with ternary lipid mixtures consisting of DOTAP, DPPC, and cholesterol, we demonstrated that phase separated liposomes yielded a 4-fold enhancement in macromolecular delivery to live cells when compared to homogenous liposomes with equivalent DOTAP concentration. When DOTAP is concentrated within one phase, that phase becomes highly fusogenic, Figure 5. By restricting the DOTAP to one region, the overall amount of DOTAP that is necessary to drive fusion is decreased. This technology serves as a starting point for the development of fusogenic liposomes that can be triggered by physical or molecular cues.

### 4.2. Targeting of Endocytosis

Nanoparticle-based drug delivery utilizes the biological and physical organization of membranes to achieve targeted effects. Recognition of the target cell type can be achieved by incorporating ligands on the nanoparticle surface that bind specific receptors on the cell membrane. Because it is a well-defined, rapid, and regulated process in mammalian cells, clathrin-mediated endocytosis is the most commonly used entry pathway (10). Nanoparticles that enter by clathrin-dependent mechanisms may display ligands such as mannose-6-phosphate or transferrin, which bind receptors destined for internalization through this route. Nanoparticle size, shape, and charge also influence the likelihood of uptake by clathrin-mediated endocytosis (115).

One obstacle to selective cell targeting arises from the fact that a unique marker for a cell type is not often abundant on the cell surface (144). Modeling nanoparticle-cell binding suggests that the key to selective cell targeting is to decorate nanoparticles with multiple ligand types that match the profile and concentrations of the cell's receptors, rather than using a single target (35). Further, concentrating ligands on the nanoparticle, either by incorporating a high density or by linking them, can drive clustering of their receptors on the cell upon binding (146, 153), Figure 5. By promoting oligomerization of endocytic

receptors and adaptor proteins, this multivalent approach also contributes to effective activation of endocytosis (146).

#### 4.3. Therapies Directed at the Immunological Synapse

The principles of membrane organization have recently been applied toward the design of materials that can modulate T cell activation by driving assembly of the immunological synapse. Specifically, micron scale particles with a stimulatory ligand on one half of their surfaces and a co-stimulatory ligand on the other half of their surfaces have been shown to have a superior ability to activate T cells compared to particles in which the two types of ligands both covered particles surfaces homogeneously (87), Figure 5. In vitro activation of T cells against specific populations of antigens over expressed in tumors has important applications in cancer immunotherapy (78).

### 5. Concluding Remarks

Here we have reviewed the major physical mechanisms by which heterogeneity in biological membranes can be stabilized. As a companion to this physical perspective, we have discussed well-known biological observations of membrane heterogeneity. For each example we have asked to what extent existing understanding of the underlying physical mechanisms is sufficient to explain the stability of the biological structure. Here it was apparent that multiple mechanisms work together synergistically to support most membrane heterogeneities found in nature. Finally we have reviewed emerging opportunities to exploit membrane heterogeneity as both a drug target and a model for engineered drug delivery systems. These examples illustrate the practical significance of ongoing efforts to understand the fundamental biophysical mechanisms responsible for membrane heterogeneity.

### Acknowledgements

This work was supported by the National Institutes of Health through F32GM128316 (Zeno), F32GM133138 (Day), R01AI121500 (Gordon), and R01GM120549 (Stachowiak). This work was also supported by the National Science Foundation through Grant 1727544 (Gordon).

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